Characterization of human duodenal fluids in fasted and fed state conditions

Danny Riethorst [a], Raf Mols [a] Guus Duchateau [b], Jan Tack [c], Joachim Brouwers [a], Patrick Augustijns [a].

a Drug Delivery and Disposition, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven, Belgium

b Unilever R&D Vlaardingen, Nutrition & Health, the Netherlands

c University Hospitals Leuven, Department of Gastroenterology, Belgium

Corresponding author:

Patrick Augustijns

Drug Delivery and Disposition, KU Leuven, Gasthuisberg O&N 2 – Herestraat 49, box 921, 3000 Leuven, Belgium;

Tel: +32 16 330301; Fax: +32 16 330305

Patrick.augustijns@pharm.kuleuven.be

1 ABSTRACT

This work provides an elaborate characterization of human intestinal fluids (HIF) collected in fasted and fed state conditions. HIF from 20 healthy volunteers (10 M/F) were aspirated by intubation near the ligament of Treitz in a time-dependent manner (10 minute intervals) and characterized for pH, bile salts, phospholipids, cholesterol, triacylglycerides (TAG), diacylglycerides (DAG), monoacylglycerdes (MAG), free fatty acids (FFA), pancreatic lipase, phospholipase A2, and non-specific esterase activity. For almost all parameters, a food induced effect was observed. Results were characterized by a high variability, as illustrated by the broad ranges observed for each parameter: pH (fasted: 3.4-8.3; fed 4.7-7.1), bile salts (fasted: 0.03-36.18 mM; fed 0.74-86.14 mM), phospholipids (fasted: 0.01-6.33 mM; fed 0.16-14.39 mM), cholesterol (fasted: 0-0.48 mM; fed 0-3.29 mM), TAG (fed 0-6.76 mg/ml), DAG (fed 0-3.64 mg/ml), MAG (fasted: 0-1.09 mg/ml; fed 0-11.36 mg/ml), FFA (fasted: 0-3.86 mg/ml; fed 0.53-15.0 mg/ml), pancreatic lipase (fasted: 26-86 µg/ml; fed 146-415 µg/ml), phospholipase A2 (fasted: 3-6 ng/ml; fed 4.3-27.7 ng/ml), and non-specific esterase activity (fasted: 270-4900 U/ml; fed 430-4655 U/ml). This comprehensive overview may serve as reference data for PBPK modeling and the optimization of biorelevant simulated intestinal fluids for the use in *in-vitro* dissolution, solubility and permeability profiling.

2 KEY WORDS

Intestinal drug absorption, Intestinal secretion, Gastrointestinal, Oral drug delivery, Food effects, Lipids, pH, Phospholipids,

3 INTRODUCTION

Variability in human drug bioavailability is often attributed to variations in hepatic first-pass extraction [1], body distribution, and kidney excretion. For those orally administered drugs, variability may be further increased by gastrointestinal factors [1], including interdigestive and postprandial gastrointestinal motility, gastric emptying [2], and human intestinal fluid (HIF) composition [3]. HIF composition is especially important for its effect on the dissolution and permeation of lipophilic drugs with a limited aqueous solubility, i.e. BCS (biopharmaceutics classification system) class II and IV

compounds [4]. Predicting the oral absorption potential of this type of drugs requires in vitro tools for dissolution, solubility and permeability, and physiologically-based pharmacokinetic (PBPK) simulation models that account for HIF composition and its related variability.

The importance of bile salts, pH, phospholipids, lipid degradation products, cholesterol, and enzymatic secretions for absorption is generally accepted. The pH is known to affect drug ionization behavior [5,6]. The surfactant properties of bile salts [7] and phospholipids [8,9] contribute to the solubilization of lipophilic compounds. After intake and enzymatic digestion of a meal, lipolytic hydrolysis products [triacylglycerides (TAG), diacylglycerides (DAG), monoacylglycerides (MAG), and free fatty acids (FFA)] form colloidal structures and are a part of mixed micelles, which impact the solubility/permeability of lipophilic compounds[10-12]. Cholesterol improves bilayer stability [13] and is incorporated in mixed micelles [14]; more recently, cholesterol has been shown to affect solubility of several BCS II class drugs [15].

As HIF is comprised of several constituents, it is prone to a large variability. In addition to inter-subject differences, day-to-day fluctuations may also lead to intra-subject variability. Moreover, the dynamic nature of the intraluminal environment, for instance mediated by meal intake and biliary/pancreatic secretions, further enhances complexity. The pronounced variability obviously complicates the definition of simulated intestinal fluids (SIF) [3]. Additionally, several limitations of the currently available literature data on HIF composition hinder a clear consensus on the simulation of HIF composition and its related variability. Data is scattered throughout numerous publications [3,16-21], all focusing on different factors and utilizing different sampling protocols. Fasted and fed state conditions are often collected on separate test days, making it impossible to discriminate between the net effect of food and the intrinsic day-to-day variability in human volunteers. Moreover, the time-dependent evolution in HIF composition is usually neglected by using only pooled samples.

This study aims to provide a comprehensive and relevant characterization of the composition of duodenal fluids and its variability. Therefore, pH, individual bile salts, phospholipids, cholesterol, lipid degradation products (TAG, DAG, MAG, and FFA), and enzymatic secretions (pancreatic lipase,

phospholipase A_2, and nonspecific esterase) were analyzed in consecutively collected fasted and fed state, time-dependent aspirates from 20 healthy volunteers. To judge the use of pooled aspirates as representative samples, volunteer pools were also created and analyzed. The data enabled insight into inter-subject and time-dependent intra-subject variability and may contribute to the use of pooled HIF samples and their derived SIF composition for physiologically-based absorption profiling.

4 MATERIALS & METHODS

4.1 MATERIALS

Taurochenodeoxycholic acid (TCDC), taurodeoxycholic acid (TDC), glycoursodeoxycholic acid (GUDC), glycochenodeoxycholic acid (GCDC), glycodeoxycholic acid (GDC), glycocholic acid (GC), chenodeoxycholic acid (CDC), deoxycholic acid (DC), lithocholic acid (LC), and cholic acid (C) were purchased from Sigma-Aldrich (St. Louis, MO). Tauroursodeoxycholic acid (TUDC), ursodeoxycholic acid (UDC), and taurocholic acid were acquired from Calbiochem (Darmstadt, Germany). Deuterated cholic acid (Cholic-2,2,4,4-$d_{4)}$ was purchased from CDN isotopes (Quebec, Canada)

NaCl, glacial acetic acid, chloroform, methanol, hexane, and pyridine were obtained from Merck (Darmstadt, Germany). The silylating agent bis(trimethylsilyl)trifluoroacetomide (BSTFA) was obtained from Pierce (Rockford, IL). The lipids DL-α-stearin, DL-1,2-dipalmitin, glyceryltripalmitin, and palmitic acid as well as the general lipase inhibitor Orlistat were obtained from Sigma-Aldrich. Water was purified with a Maxima system (Elga Ltd, High Wycombe Bucks, UK). Since solid meals are incompatible with the aspiration of intestinal fluids (clogging of catheter), the liquid meal Ensure Plus (Abbott Laboratories B.V., Zwolle, The Netherlands) was used to simulate a standard meal. One portion of 200 mL has an energy content of 300 kcal of which lipids, carbohydrates and proteins constitute 29%, 54%, and 17%, respectively

4.2 SAMPLING OF HIF

The sampling of the intestinal fluids was performed at the University Hospitals Leuven and was approved by the Committee of Medical Ethics (ML7918). Twenty healthy Caucasian volunteers were

enrolled in the study after giving written informed consent. The volunteers were selected as in a typical bioavailability/bioequivalence study. Ten men and ten women, aged between 18 and 31 years and with a BMI between 19 and 25 kg/m^2, participated in the study. None of the volunteers had a history of gastrointestinal diseases and medication was omitted for 2 days before participation in the study. After an overnight fast, one double-lumen catheter (Salem Sump Tube 14 Ch, external diameter 4.7 mm, Sherwood Medical, Petit Rechain, Belgium) was introduced in the duodenum (D2–D3) via the nose or mouth and the position was checked fluoroscopically (Figure 1A). This double-lumen catheter enabled the collection of intestinal fluids by means of a syringe without creating underpressure in the gastrointestinal (GI) tract.

After an overnight fast of at least 12 hours (no food and only water), the volunteers were given 250 ml of water before initiating the sampling of fasted state intestinal fluids. Following the fasted state, 400 mL of Ensure Plus was ingested to simulate a standard meal; this condition is referred to as the fed state. A glass of 250ml water was consumed 20 minutes after intake of the liquid meal (Figure 1B). After the ingestion of water (both for fasted and fed state), intestinal fluids were sampled every 10 min for a period of 90 min. In total, 18 time-dependent samples were obtained per healthy volunteer; sample volumes did not exceed 10ml per time-point. Intestinal samples were collected in test tubes containing the lipase inhibitor Orlistat dissolved in ethanol to arrest further lipolysis in vitro. A stock solution of 1 mM Orlistat in ethanol was used to bring the final concentration of Orlistat in the duodenal sample to 1 µM. This protocol allowed minor dilution of the collected samples as well as negligible addition of ethanol (0.1%, v/v). The applied concentration of this lipase inhibitor is 100-fold the IC50 (11 nM), as determined previously (Unilever R&D, Vlaardingen, data not published).

4.3 HANDLING OF SAMPLES

All aspirated fluids were kept on ice in closed test tubes till the end of sampling, followed by a 15-min centrifugation at 1500 x g and 4 °C. All samples were stored at -20 °C until further analysis. Aliquots (<1ml) of each sample were stored for characterization. The remainder of the collected fractions was pooled for each volunteer in fasted and fed state (further referred to as volunteer pools). Pools were

created immediately after the collection was complete. For a selected set of samples, separate aliquots without Orlistat were collected in order to measure esterase activity; additionally, protease inhibitors were added to aliquots of another set of samples used for lipase quantification.

4.4 ANALYSIS OF SAMPLES

4.4.1 pH

The pH of all samples (see Sampling of HIF Section) was measured immediately upon collection (Hamilton Slimtrode); the pH of the volunteer pools was measured immediately after pooling.

4.4.2 PHOSPHOLIPIDS

Total phospholipid levels were determined enzymatically employing the Phospholipids FS kit from DiaSys (Holzheim, Germany). After release by phospholipase D, choline is oxidized by choline oxidase, which generates hydrogen peroxide. Subsequently, peroxidase forms a quinone that was analyzed spectrophotometrically at 570 nm [22] using a Tecan Infinite M200 plate reader (Tecan Group Ltd., Männedorf, Austria). The assay was used in a 96-well format with a detection limit of 0.1 mM. Biologically relevant bile salts (TC, TCDC, TDC, GC, GCDC, and GDC) at concentrations of 10 mM each (and combined 60 mM) did not interfere with the assay.

4.4.3 CHOLESTEROL

Total cholesterol was determined enzymatically employing an Amplex® Red cholesterol assay kit from Molecular Probes Invitrogen (Eugene, Oregon, United States). Cholesterol esters are hydrolyzed to free cholesterol by cholesterol esterase. Subsequently, cholesterol is oxidized by cholesterol oxidase to yield hydrogen peroxide. In the final step, hydrogen peroxide reacts with Horse radish peroxidase and Amplex® Red to form resofurin [23]. This reaction product was measured using fluorescence (Tecan Infinite M200 plate reader) with an excitation/emission at 560 nm/590 nm, respectively. The detection limit of the assay was 200 nM.

4.4.4 BILE SALTS

Bile salts were analyzed using a Thermo Fisher Scientific LC-MS/MS system. This system consisted of a TSQ Quantum Access™ triple quadrupole mass analyzer, equipped with an electrospray ionization (ESI) source, and coupled to an Accela™ U-HPLC system (San Jose, CA, USA). Data acquisition and peak integration were performed with the Xcalibur® 2.0.7 and LCquan® 2.5.6 software packages, respectively. For separation, a Kinetex C18 column (1.7 μm, 100 A, 50 × 3 mm) with an in-line KrudKatcher ultra HPLC filter (PhenomenexR, the Netherlands) was used and the column temperature was kept at 30 °C. The injection volume was 25 μl using a full loop. The flow rate was 450 μl/min and bile salts were eluted using a gradient shown in Table 1A, with the buffer consisting of 5 mM ammonium acetate (pH adjusted to 3.5 with acetic acid). Samples were diluted 1000-10000 times in 50/50 MeOH/H_2O containing 200 nM deuterated cholic acid (D4C) as internal standard. Analysis was performed using the negative electrospray ionization mode in combination with the following parameters: capillary temperature 275 °C, vaporizer temperature 300 °C, sheath gas (N_2) pressure 40 (arbitrary units), auxiliary gas (N_2) pressure 45 (arbitrary units), ion sweep pressure 20 (arbitrary units), spray voltage 3500 V, and collision gas (Ar) 1.5 mTorr. The parent and daughter mass of each bile salt are shown in Table 1B. Three groups of bile salts have identical mass and daughter ions but could be separated via HPLC. Furthermore, UDC, CDC, DC, and LC could not be fragmented and, therefore, the parent mass was analyzed. Total bile salt concentrations were determined as the sum of individual bile salts. The calibration curve was made in 50/50 MeOH/H_2O (200 nM D4C) and was linear between 0.009 μM and 5 μM.

The method validation showed an inter-day repeatability in a HIF standard sample (randomly selected) of <10.5% RSD (relative standard deviation) for the individually determined bile salts; the inter-day RSD for the total bile salt concentration was 5.4%. Samples containing 500 nM or 50 nM (in MeOH) of each individual bile salt showed a RSD of <7.9% or < 11%, respectively. The concentration determined in the HIF standard (stored at -20°C) remained stable for > 12months. The intra-day RSD

for individual and total bile salt concentrations in the HIF standard was <8.8%, and 3.6%, respectively. Samples containing 500 nM or 50 nM displayed an intra-day RSD of <6.0% and <10.7%, respectively. During each analysis, 4 quality control (QC) samples (50nM, 500nM, 2000nM, and a HIF standard) were determined at least 5 times spread over the analysis (1 set of controls every 15-25 samples). All QC samples displayed accuracy between 90-110% and RSD <10%, for each individual bile salt.

4.4.5 LIPID CONTENT

After freeze-drying of a 100µl HIF sample, lipids were extracted consistent with the method of Armand *et al*.[24] Briefly, the freeze dried samples were dissolved in 0.15 M NaCl containing 2% glacial acetic acid. Chloroform/methanol (2:1, v/v) was used as extraction solvent. The lower chloroform layer was evaporated to dryness under a stream of nitrogen. The residue was silylated using BSTFA (20% in pyridine, v/v). After 30 min at 60°C, the sample was dissolved in hexane and analyzed by GC [Fisons MFC800] with flame ionization detection according to the technique described by Duchateau *et al*.[25] The column used was a Chrompack, CP-Sil5CB-MS column with a deactivated fused silica precolumn. Samples were injected ‘‘cold-on-column’’ and were heated to 360°C with a rate of 10°C/min. Hydrogen was used as carrier gas. The injection volume was 1 µl and total run time was 50 min. Lipids were separated according to carbon number (CN). For our purpose, peaks were integrated individually and combined to obtain one AUC (area under the curve) value for each class of neutral lipids. Peaks in the range of CN 8–18, CN 20–28, CN 30–42, and >CN 44 were assigned to free fatty acids (FFA), monoacylglycerides (MAG), diacylglycerides (DAG), and triacylglycerides (TAG), respectively (using calibration standards of the respective classes). These integration ranges were chosen to minimize overlap between the different lipid classes. The amount of each class was calculated using the respective calibration curve: palmitic acid, mono-palmitin, di-palmitin, and tripalmitin standards were dissolved in hexane and appropriate volumes were evaporated under nitrogen to obtain known amounts of the respective standards. The standards were extracted and silylated identically to the unknown samples.

4.4.6 SPECIFIC ESTERASES: PANCREATIC LIPASE & PHOSPHOLIPASE A_2

Both pancreatic lipase and phospholipase A_2 quantities were determined using a commercially available competitive ELISA from Bluegene (Shanghai, China). Separate aliquots were stored at -20°C and only thawed a maximum of 2 times. Samples were diluted in PBS (pH7.4) containing 5% (v/v) protease inhibitor cocktail including AEBSF at 104 mM, aprotinin at 80 µM, bestatin at 4 mM, E-64 at 1.4 mM, leupeptin at 2 mM and pepstatin A at 1.5 mM.

4.4.7 NON-SPECIFIC ESTERASE ACTIVTIY

Esterase activity was assayed by measuring the conversion of p-nitrophenyl acetate to p-nitrophenyl which could be analyzed spectrophotometrically at 410 nm [26] (Tecan Infinite M200 plate reader). Stock solutions of both of p-nitrophenyl acetate and p-nitrophenyl were prepared in CH_2Cl_2 at concentrations between 100 and 250 mM. Enzymatic activity was measured in 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 0.01% Gum Arabic at 25 °C for 30 minutes. HIF samples of 5 µL were incubated with 0.2 mL substrate solution in 96-well clear microtiter plates. Substrate was added to the reaction buffer at a final concentration of 0.5 mM. Unit (U) definition: *One unit will hydrolyze 1.0 µmole of p-nitrophenyl-acetate to p-nitrophenyl per minute at pH 8.0 at 25 °C.* For this assay, aliquots of 500 µL HIF were separated before addition of Orlistat to avoid inhibition of esterases. These separate aliquots were generated for 10 volunteer pools and for time-dependent samples of 3 volunteers.

5. RESULTS & DISCUSSION

In this study, human intestinal fluids were collected as a function of time from 20 volunteers in fasted and fed state conditions. All HIF aspirates were characterized for pH, bile salts, phospholipids, cholesterol, and lipid hydrolysis products, all of which may directly affect drug solubility and/or permeability. Additionally, enzymes were assessed that may indirectly affect drug absorption: secretion of pancreatic lipase and phospholipase A_2 influences the intraluminal environment through lipid and phospholipid degradation; esterase activity plays an important role in the conversion of ester prodrugs. Data are presented both as individual data points and in a time-dependent way.

5.1 INDIVIDUAL DATA POINTS

In each volunteer, HIF aspirates were characterized at, on average, 17 out of a possible 18 time points, resulting in a total of 161 and 175 individual data points for fasted human intestinal fluids (FaHIF) and fed human intestinal fluid (FeHIF), respectively. These individual data points are presented in Figure 2 providing insight into the overall duodenal variability in pH, bile salts, phospholipids, cholesterol, and lipid degradation products. This overview clearly shows that some factors are asymmetrically distributed, with mean values surpassing the more appropriate median values. It should be noted that such a distribution complicates the comparison of results with literature as most literature data is based on pooled values and/or average time profiles.

5.1.1 pH

In the fasted state, highly variable pH values were observed, ranging from 3.4 to 8.3 (Figure 2A). The mean value amounted to 6.78 and the median value to 6.88. These values are slightly higher than the pH defined for FaSSIF (pH 6.5), but still comparable to literature values (between pH of 6-7 [3,18]). pH displays a symmetrical distribution; for the majority of the samples, a pH between 6.5 and 7.5 was observed. However, pH values below 6.0 or above 7.5 were not unusual. In fed state conditions, a more narrow symmetrical distribution in pH values was observed (between 4.7 and 7.1). The observed mean (6.22) and median (6.28) pH values correspond closely to the median of pH 6.3 reported in literature [3]. These values are higher than the pH of FeSSIF (5.0) or FeSSIF-v2 (pH 5.8). The reduced variability in fed state conditions might be caused by the buffer capacity of food. Ensure Plus has a pH of 6.6 and buffer capacity 20-24 mmol/L/ΔpH [27].

5.1.2 BILE SALTS

As illustrated in Figure 2B, bile salt concentrations are characterized by a large variability and an asymmetric distribution. Total fasted state bile salt concentrations range from 0.03 mM to 36.18 mM. The majority of values are clustered below 5 mM. Due to some extreme bile salt concentrations, the mean total concentration (4.61 mM) exceeds the median value (3.30 mM). Both mean and median are within the reported average range of 1.4-8.1 mM [3,18]; the median value observed in this study is similar

to the median value of 3.25 mM reported by Bergstrom *et.al* [3] and corresponds to the concentration of sodium taurocholate used in FaSSIF (3 mM). The range observed for fed state conditions was even larger, with values ranging from 0.74 to 86.14 mM. A sizable difference was observed between the mean (12.65 mM) and median (9.59 mM) concentration. Both values are within the wide range of average values reported in literature (between 3.6-24.0 mM) [3]; the median concentration of 11.8 mM reported by Bergstrom *et.al* is in between the observed mean and median. The observed median concentration is well reflected in FeSSIF-V2 (10 mM), whereas for standard FeSSIF, a higher concentration of bile salts (15 mM) is used.

Variability was not only observed for total bile salt concentrations, but was also reflected in the composition of the individual bile salts between volunteers, as shown in Figure 3A. Each symbol in Figure 3A represents the average of all time-points for a single volunteer. Interestingly, the relative abundance of individual bile salts remained nearly constant over time for all 20 healthy volunteers (illustrated for 4 volunteers in Figure 3B), and no differences could be observed between fasted and fed state. The observed composition is largely in agreement with other studies [18,21,28]. When considering conjugated bile salts, glyco-conjugated bile salts (70%) appeared to be more abundant than tauro-conjugated bile salts (30%); others have reported higher percentages of tauro-conjugates in HIF collected from the duodenum [3,20], which might be explained by population differences as we also observed a higher abundance of tauro-conjugates in a few volunteers.

5.1.3 PHOSPHOLIPIDS

In fasted state conditions, phospholipids display a similar asymmetric distribution as bile salts with values ranging from 0.01 mM to 6.33 mM (Figure 2C). Because of extremes, the mean value (0.95 mM) exceeds the median (0.58 mM). In literature, a wide range of FaHIF phospholipid concentrations have been reported (0.1-1.8 mM [18]); both mean and median values are well within this range. The concentration used in FaSSIF (0.75 mM) is in-between mean and median. The variability observed in this study may also explain why some studies report low mean concentrations (0.1-0.5 mM [18,29]) while others report higher values [30,31]. However, the clustering between 0.1 and 0.5 mM observed in this

study supports the lower values reported most frequently in literature [3,18]; this lower value has also been adapted into FaSSIF-v2 (0.2 mM). In contrast, phospholipid concentrations measured in FeHIF displayed a more symmetrical distribution which results in nearly identical values for mean (4.17 mM) and median (4.05 mM). Still, phospholipid concentrations in fed state conditions ranged from 0.16 mM to 14.39 mM. These average values are better reflected in FeSSIF (3.75 mM) than in FeSSIF-v2 (2mM). In literature, average postprandial phospholipid concentrations are reported between 1.2 and 6.0 mM with a median of 2.15 mM [3]; however, this study confirms a tendency towards higher values reported around 4 mM [3].

5.1.4 CHOLESTEROL

In FaHIF, cholesterol is only present at low concentrations ranging between 0.00 mM and 0.48 mM (Figure 2D). As such, there is little difference between mean (0.07 mM) and median (0.08 mM). These values are equal to the 0.08 mM reported by Persson *et al* [28]; others have reported average fasted state cholesterol concentrations between 0.17 and 0.23 mM [31,32]. A higher concentration of cholesterol in FaHIF (1.8 mM) was reported by Heikkila *et al*; that study also noted there was no difference with cholesterol concentrations in FeHIF (2.0 mM). In the present study, however, both cholesterol concentrations and variability clearly increased after intake of the liquid meal, with an observed range from 0.00 mM to 3.29 mM. The presence of high extremes resulted in a mean (0.71 mM) that moderately exceeds the median (0.57 mM). In literature, data about postprandial cholesterol concentrations are limited. However, the average of 0.9 mM reported by Vertzoni *et al* [33] is close to our observations. It should be noted that the cholesterol content in Ensure plus amounted to 0.45 mM. Taking into account the dilution by water intake (250ml), chyme, and intestinal secretions, it may suggest that endogenous secretion of cholesterol via bile increases considerably in fed state conditions.

5.1.5 LIPIDS

In FaHIF, the only lipid classes observed are FFA and MAG (Figure 2E), with the majority being FFA C16/C18; hydrolysis of phospholipids may be the source of FFA in fasted state as they mainly consist of C16/C18 chain lengths. Fasted state FFA concentration displayed a symmetrical distribution with

the bulk of values between 0 and 1 mg/ml; only a few outliers were observed resulting in an absolute range from 0 - 3.86 mg/ml. Consequently, mean (0.53 mg/ml, ≈2.07 mM) and median (0.45 mg/ml, ≈1.75 mM) FFA concentrations are nearly equal and consistent with reported literature values between 0.4 and 0.6 mg/ml [24,30]. If present, MAG concentrations in FaHIF were generally very low. Values ranged between 0 mg/ml and 1.09 mg/ml (≈3.30 mM); however, only one sample exceeded 0.43 mg/ml (1.30 mM). The mean (0.13 mg/ml, ≈0.39 mM) and median (0.10 mg/ml, ≈0.30 mM) MAG concentrations in FaHIF are nearly identical. In literature, the only reported MAG concentrations amount, on average, to 0.20 mg/ml [30].

Postprandial lipid concentrations are obviously mainly determined by meal intake. The Ensure plus lipid content was determined to be 53 mg/ml (49 mg/ml according to manufactures specification), of which 92% consisted of TAG. In line with literature data [3,21,24,30], the majority of lipids in fed state duodenal fluids are degradation products in the form of FFA and MAG. The largest fraction in FeHIF was, by far, FFA C18. Obviously, the chain length distribution will depend on the type of food, and thus on the TAG present. For both FFA and MAG concentrations, a rather broad distribution was observed: FFA displayed a range between 0.53 and 15.0 mg/ml (≈2.1-58.7 mM), while MAG ranged between 0 and 11.36 mg/ml (≈0-34.4 mM). FFAs are by far the largest factor in FeHIF with a mean and median of 6.49 mg/ml (≈25.4 mM) and 5.63 mg/ml (≈22.0 mM), respectively. These values are lower than reported average FFA concentrations of 39.4 and 52.0 mM [19,33]. The observed mean (3.05 mg/ml, ≈9.24 mM) and median (2.75 mg/ml, ≈8.33 mM) MAG concentrations in FaHIF are in-line with MAG concentrations reported in literature (between 5.9 and 8.1 mM). In all postprandial aspirates, advanced lipid degradation was seen, resulting in relatively low DAG and TAG concentrations. Fed state DAG and TAG concentrations were absent in a large amount of aspirates causing an asymmetrical distribution between 0.00 and 3.64 mg/ml, and between 0.00 and 6.76 mg/ml, respectively. Consequently, a substantial difference was observed between mean (0.61 mg/ml, ≈1.04 mM) and median (0.36 mg/ml, ≈0.61 mM) DAG concentrations, as well as between mean (0.81 mg/ml, ≈0.96 mM) and median (0.59 mg/ml, ≈0.70 mM) TAG concentrations.

5.2 TIME-DEPENDENT EVOLUTION

The results so far illustrate that the intestine is a highly variable environment, which will definitely impact drug and formulation behavior. As part of this overall variability, several parameters fluctuate as a function of time, as demonstrated in Figure 4 which displays average values for the 20 healthy volunteers. Especially after intake of the meal (at 100 min), a clear time-dependent evolution is seen for pH, bile salts, phospholipids, cholesterol and lipid degradation products (food effect). For none of these factors, fasted state conditions were restored after 90 minutes of sampling in the fed state.

In the fasted state, slightly lower pH values were observed during the first 40 min, which may be attributed to the initially fast transfer of (acidic) gastric contents upon intake of a glass of water (Figure 4A). On average, pH fluctuates around 6.5, being slightly higher in FaHIF as compared to FeHIF. Typically, pH varies more in the fasted state (larger SEM) than in the fed state, which was already demonstrated in Figure 2. Average concentrations of bile salts (Figure 4B) and phospholipids (Figure 4C) appear to be relatively stable in the fasted state. Postprandial concentrations increase and are characterized by higher variability. This variability may be attributed to the fact that the release of bile is not continuous, but rather pulsatile. Differences in timing and intensity of these contractions result in strong fluctuations as a function of time and, in some individuals, extreme values (for instance bile salt concentrations up to 86 mM). It is worth mentioning that the ratio of bile salt concentrations over phospholipid concentrations (Figure 4D) remained impressively constant in fed state conditions. This contrasts with this ratio observed in fasted state conditions. Cholesterol (Figure 4E) enters the duodenum through food intake but especially through bile secretions, thus resulting in a similar profile as observed for bile salts and phospholipids.

As expected, little variation was observed in the basal levels of lipid products in fasted state conditions (Figure 4F). Postprandially, FFA and DAG but not TAG and DAG significantly increase (as already demonstrated in Figure 2). The majority of lipid species are FFA, which implies a very rapid and effective conversion of TAG into FFA and MAG. It is also interesting to see that total duodenal lipid concentrations appear to be tightly controlled by gastric emptying, as they rarely exceed 15 mg/ml, in

agreement with the caloric-dependent gastric release [34]. This also corresponds with a previous study by Clarysse *et al.*[30] in which both Ensure Plus and a fat-enriched meal were administered; the higher lipid content in the fat-enriched meal did not result in higher duodenal lipid concentrations as a consequence of this caloric brake [34,35].

Finally, it should be noted that food effects on HIF composition will strongly depend on the type and composition of the administered meal. For instance, as compared to the liquid meal used in the present study, solid meals will have a different effect on gastric motility and emptying, most likely resulting in a different time-dependency of the food effect.

5.3 VOLUNTEER POOLS

In view of the limited volume collected during sampling of HIF, aspirates from different volunteers are usually combined when using HIF for dissolution, solubility, or permeability studies. In the present study, no general 'population' pool was generated. However, volunteer pools were created by combining aliquots of the time-dependent aspirates per volunteer. For each volunteer, a fasted and fed state pool were made, each representing the average HIF aspirated during the respective 90 min time periods. In Figure 5, the composition of all volunteer pools is represented, demonstrating high inter-subject variability for most factors. Despite an unequal distribution, the pool-derived means (Figure 5) correspond in general to the mean values obtained from all individual data points (Figure 2). The data presented in Figure 5 illustrate that food effects may significantly vary between volunteers: for bile salt concentrations, for instance, food intake resulted in a 20-fold increase in some volunteers (e.g. HV 6), while for others almost no increase (e.g. HV 5), or even a decrease (e.g. HV 18), was observed.

Considering the high variability observed among the different volunteer pools, it is clear that population pools created from different volunteers can only be useful to evaluate the average effect of HIF on dissolution, solubility and permeation. The use of volunteer pools is critical to explore inter-subject differences in drug and formulation performance.

5.4 ENZYME SECRETIONS

The parameters discussed so far all have a direct effect on the solubilizing capacity of HIF; however, indirect factors that shape the intestinal environment (e.g. lipases) may pose an additional source of variability in absorption. In addition, intestinal esterases may hydrolyze orally dosed ester prodrugs, which can lead to variations in pharmacokinetic and pharmacodynamic responses to these compounds. As shown in Figure 6 (A-C), HIF from 10 individual volunteers (pooled samples) were studied for pancreatic lipase, phospholipase A2 and esterase. For 3 volunteers, time profiles were included (Figure 6 D-F).

5.4.1 PANCREATIC LIPASE

Gastric (5–40%) and pancreatic lipase (40-70%) mediate the degradation of lipids and may, as such, exert an important indirect effect on intestinal drug absorption [16]. As indicated in Figure 6, fasted state pancreatic lipase concentrations ranged between 23 and 86 µg/ml (mean = 49 ± 21 µg/ml). Pancreatic lipase is reported to have a specific activity of 8000 IU/mg [16], which means that fasted state HIF has an activity between ≈184 and 690 IU/ml. Postprandially, pancreatic lipase concentration increased 5-10 fold, which is comperable to a 5-fold increase reported by Keller *et. al* [36]. The observed range from 146 µg/ml (≈1170 IU/ml) to 415 µg/ml (≈3320 IU/ml), with an average of 293 ± 93 µg/ml (≈2350 IU/ml), was in line with reported values between 600-7000 IU [16]. The lack of DAG and TAG at all time-points indicates a potent degradation of TAG by lipases present in the GI tract. Figure 6-D illustrates that, postprandially, pancreatic lipase is secreted in a similar manner (contraction-wise) as other secretions from the papilla of Vater (e.g. bile salts and phospholipids).

5.4.2 PHOSPHOLIPASE A_2

Phospholipids and lyso-phospholipids each have distinct properties with respect to absorption because lyso-phospholipids are stronger surfactants [9,37,38]. Phospholipase A_2 mediates the conversion to the lyso form. Several studies have shown that nearly 90% of phospholipids present in duodenal fluids are lyso-phospholipids [21,28]. Despite their reported effect on the absorption of vitamin A/E and cholesterol [14,37], little is known about duodenal phospholipase A_2 (PLA_2) concentrations (only using secretin stimulation [39]). In fasted state, PLA_2 concentrations ranged between 3 and 6 ng/ml (mean = 4.3 ± 1

ng/ml), which increased approximately 5-fold in fed state conditions with an observed range between 4.3 ng/ml and 27.7 ng/ml (mean = 14.1 ± 7.8 ng/ml). Figure 6-E illustrates that PLA_2 concentrations increase, on average, in a similar proportion and contraction-like manner as phospholipid concentrations in Figure 4C (4-5 fold).

5.4.3 ESTERASE ACTVITY

The rate of prodrug conversion can indirectly influence the absorption of prodrug or parent drug [40-42]. As such esterase activity can introduce another factor of variability for the absorption of orally dosed ester-prodrugs. For the measurement of esterase activity, no Orlistat was added during the collection of the samples as it can inhibit esterase activity [43]. The activity assay is based on conversion of a ‘’small ’’ acetate ester, as larger esters are generally converted by specific esterases (e.g. lipases) [26]. Figure 6 C and F show that, in contrast to lipases, esterases do not differ between fasted (270-4900 U/ml) and fed state (430-4655 U/ml). Non-specific esterases such as carboxyl esterase are expressed in almost all tissues including the duodenum [42]. Hence, concentrations are not only influenced by pancreatic secretions and might even be higher near the intestinal brush border.

6 CONCLUSION

This work has generated a comprehensive overview of human intestinal fluid composition with an unparalleled insight into its time-, food- and subject-dependent variability. As such, it provides a solid data-set for future development of biorelevant absorption profiling tools. Considering the high interindividual variability, the use of averages might be misleading when simulating HIF composition. None of the pooled samples mimicked the total average composition that was observed. Therefore, in addition to ‘’average’’ SIF it might be useful to generate SIF for high and low responders, as well as time-dependent variations, to investigate potential effects on drug dissolution, solubility and permeability. Incorporating the observed variations in HIF composition into physiology- and population-based simulation tools will be a vital step towards the improved prediction of variability in intestinal drug absorption.

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8 FIGURES

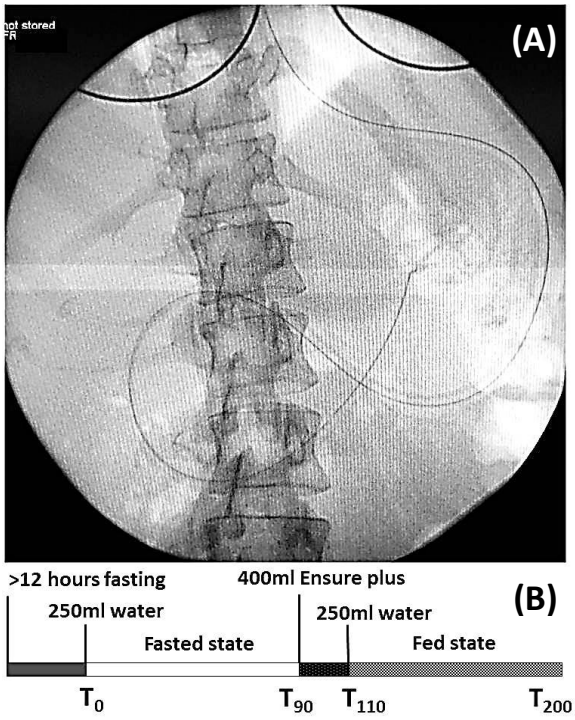
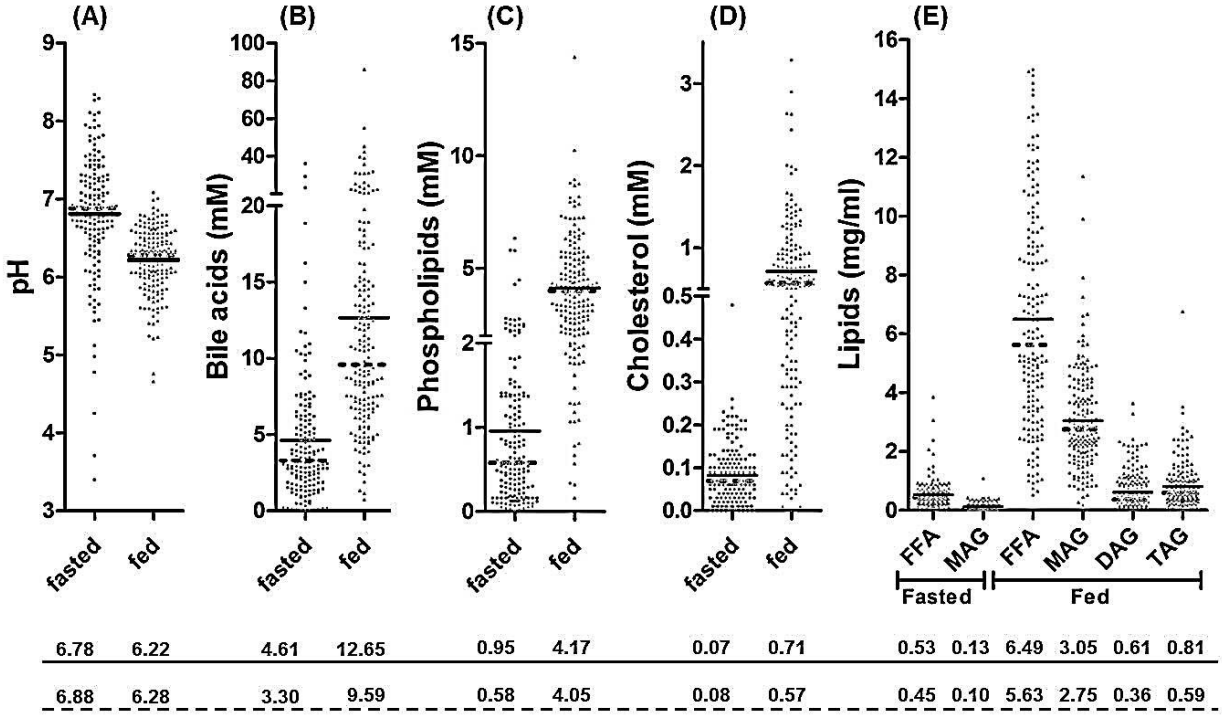


Figure 1: (A) A double-lumen catheter is positioned near the ligament of treitz. (B) Sampling protocol over time. Sampling was performed every 10 minutes for a period of 1.5h in both fasted and fed state. T0 and T110 were not included. Between T90 and T110 was a resting period.



6.78	6.22	4.61	12.65	0.95	4.17	0.07	0.71	0.53	0.13	6.49	3.05	0.61	0.81
6.88	6.28	3.30	9.59	0.58	4.05	0.08	0.57	0.45	0.10	5.63	2.75	0.36	0.59

Figure 2: Overall variability in the composition of HIF aspirated as a function of time from 20 volunteers, presented as individual data points. Mean and median values are displayed using a solid or dashed line, respectively. The exact values for mean (above the solid line) and median values (above the dashed line) are displayed below their respective graphs, in the same unit of measurement as used in the graph.

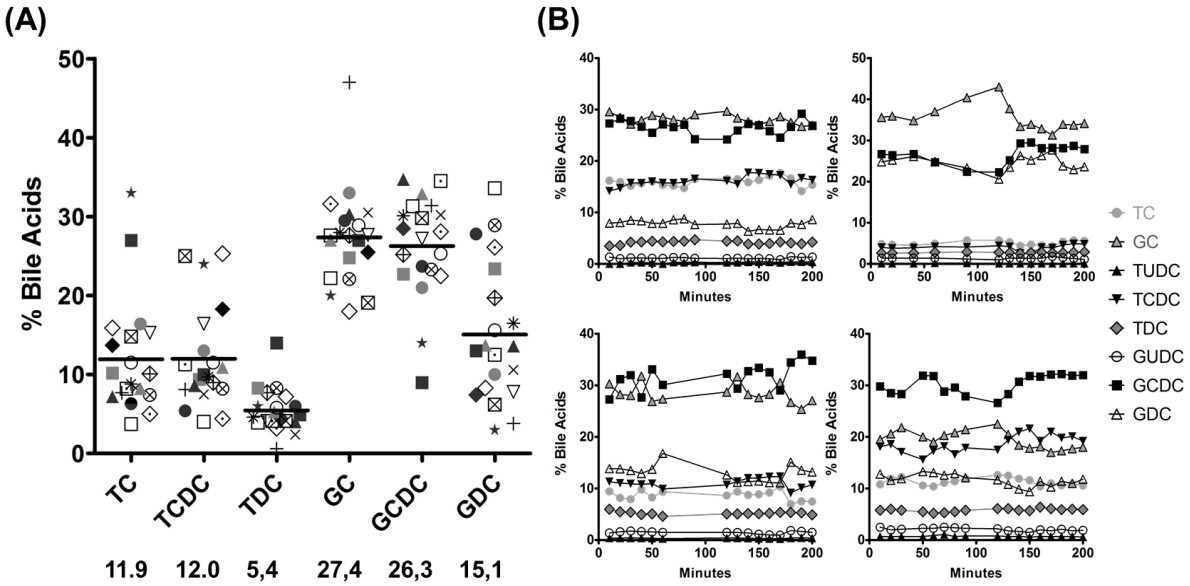


Figure 3: (A) Bile salts composition in healthy volunteers (HV). Each symbol represents the average bile acid composition of 1 HV. The solid line represents the mean value. The average percentages for TC, TCDC, TDC, GC, GCDC, and GDC are displayed below the graph. TUDC and GUDC are not included in the figure because of their minimal occurrence and variation; however, they complete the composition with, on average, 0.3% and 1.6%, respectively. (B) Examples of the bile acid composition as a function of time in 4 representative volunteers.

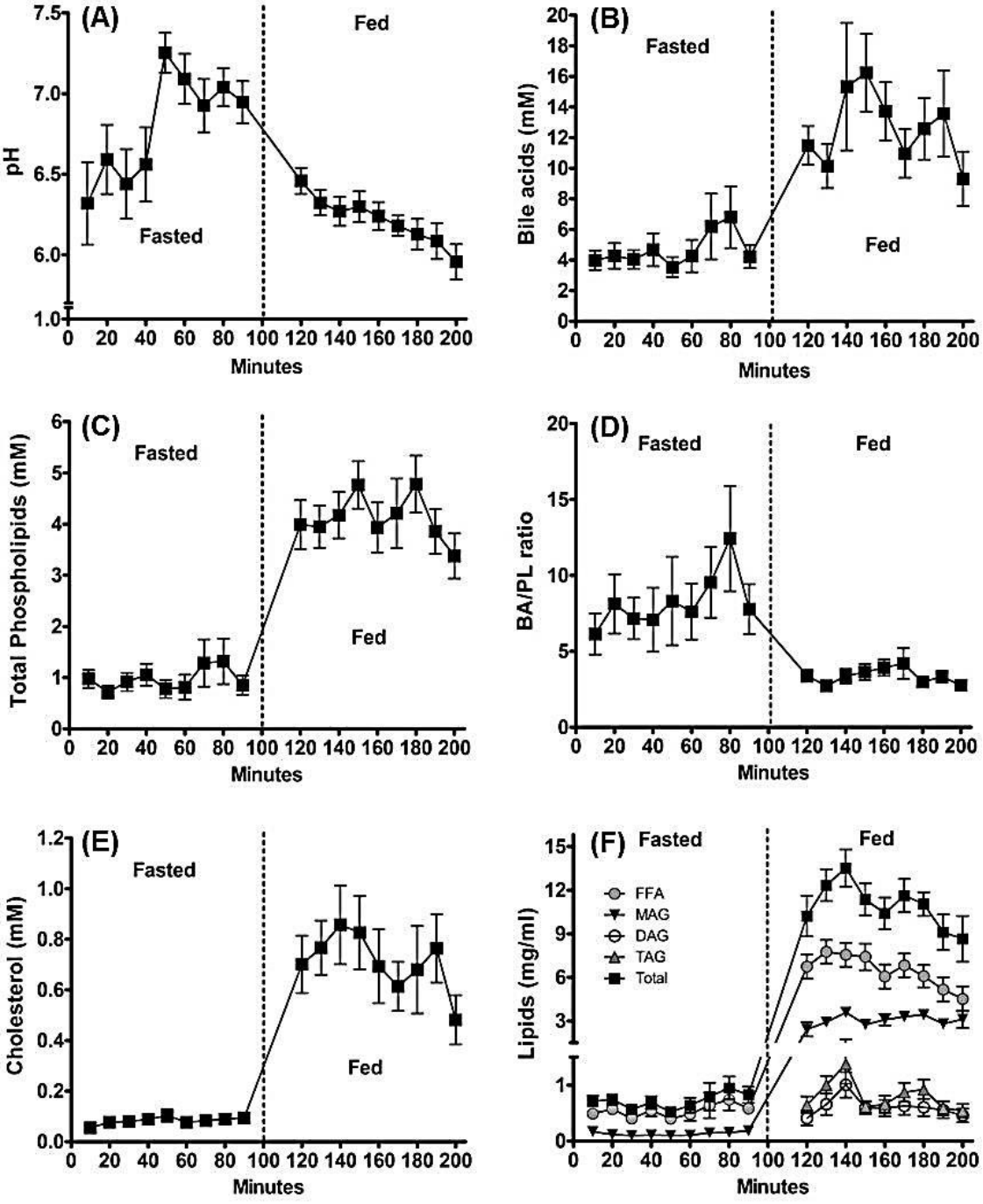


Figure 4: Time-dependent evolution of (A) pH, (B) Bile salts, (C) Phospholipids, (D) Bile acid over Phospholipid ratio, (E) Cholesterol, and (F) Lipid products. Data are presented as the mean of 20 volunteers (n = 20) ± standard error of mean. For time points at which samples could not be collected for all volunteers, n < 20 (but always n ≥ 17).

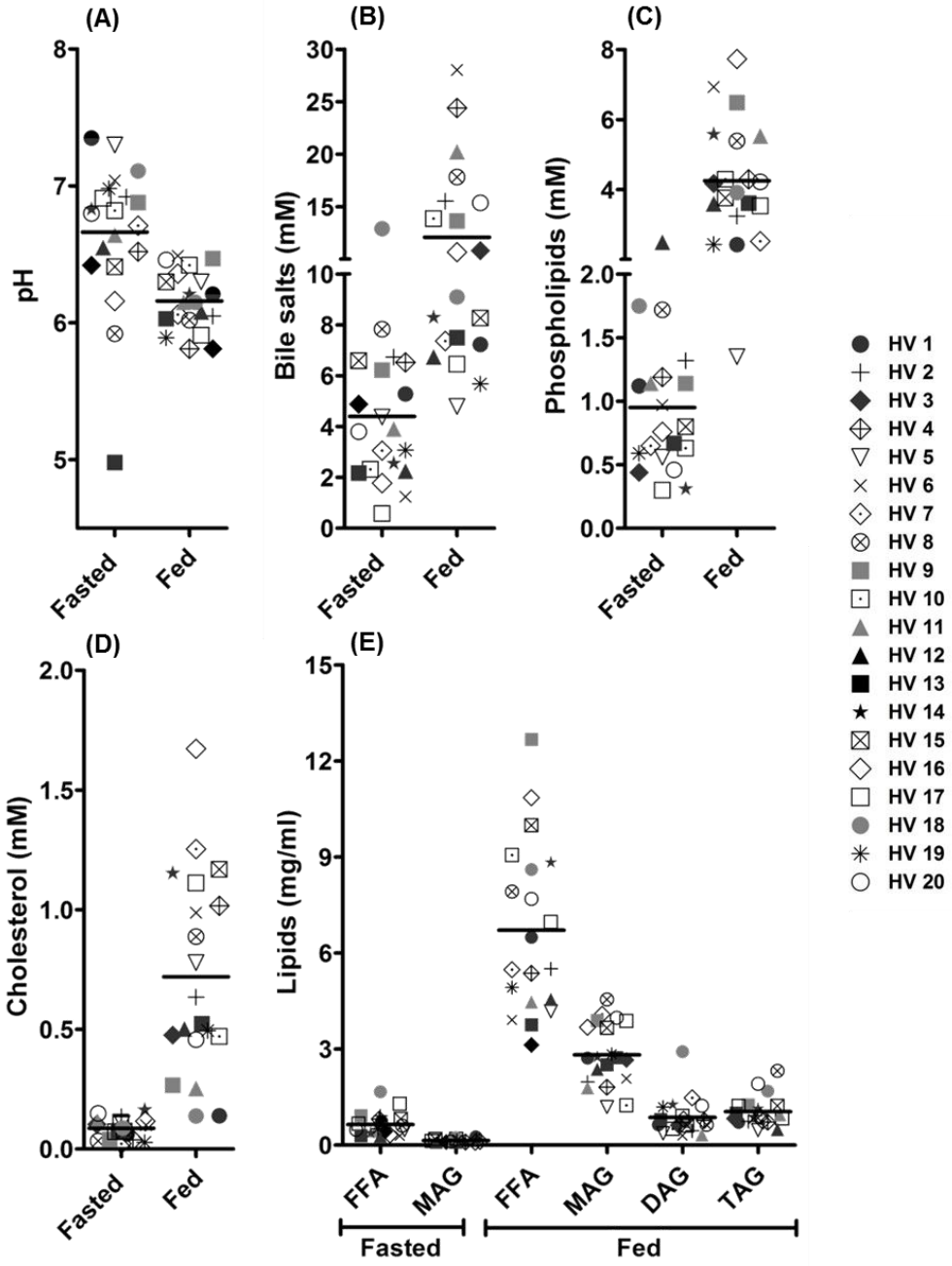


Figure 5. Composition of volunteer pools: (A) pH (B) Bile salts (C) Phospholipids (D), Cholesterol (E) Lipids/ Lipid degradation products. Volunteer pools were created by combining aliquots of the time-dependent aspirates per volunteer. Each symbol represents a volunteer. The solid line represents the mean value.

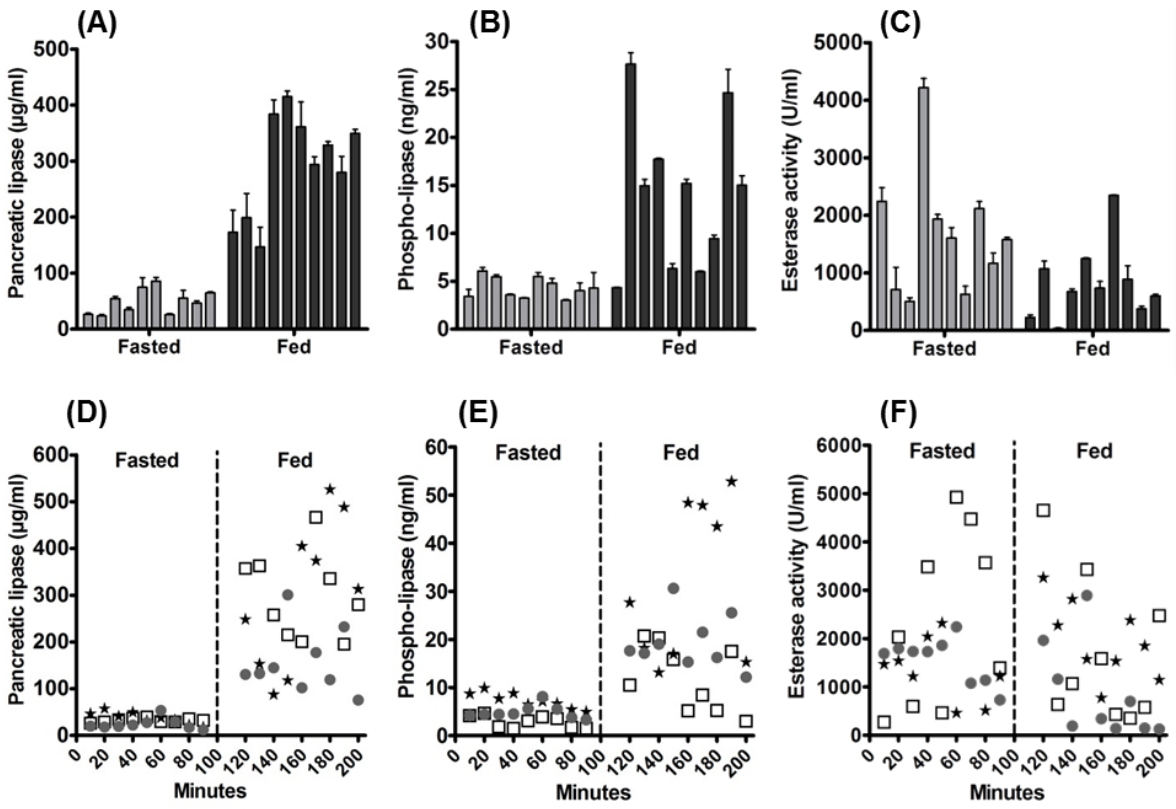


Figure 6: Enzymes in HIF: (A) pancreatic lipase quantities for 10 individual volunteers, (B) Phospholipase quantities for 10 individual volunteers, (C) Total non-specific esterase activity for p-nitrophenylacetate, (D) Pancreatic lipase quantities over time for 3 independent volunteers, (E) Phospholipase quantities over time for 3 independent volunteers, (F) Esterase activity over time for 3 independent volunteers. Pancreatic and phospholipase were determined in the same set of volunteers. For esterase activity separate samples without Orlistat were used.

Table 1: Analysis of bile salts using LC-MS/MS: (A) HPLC gradient and (B) parent and product mass used for detection

(A) HPLC gradient

Time	Acetonitrile %	Methanol %	H_2O %	Buffer %
0.0	0.0	5.0	91.0	4.0
0.5	0.0	5.0	91.0	4.0
1.0	13.0	48.0	35.0	4.0
5.0	13.0	48.0	35.0	4.0
7.0	19.0	48.0	29.0	4.0
8.9	19.0	48.0	29.0	4.0
9.0	33.0	48.0	15.0	4.0
10.4	33.0	48.0	15.0	4.0
10.5	0.0	5.0	91.0	4.0
12.0	0.0	5.0	91.0	4.0

(B) Mass

	Parent	Daughter	Collision energy
TUDC-TCDC-TDC	498.258	124.05	52
GUDC-GCDC-GDC	448.275	74.250	43
TC	514.257	124.05	52
GC	464.269	74.250	41
UDC-CDC-DC	391.256	---	0
C	407.252	343.252	37
LC	375.228	---	37
D_4C (IS)	411.286	347.153	0

9 REFERENCES

1. Kostewicz ES, Aarons L, Bergstrand M, Bolger MB, Galetin A, Hatley O, Jamei M, Lloyd R, Pepin X, Rostami-Hodjegan A, Sjogren E, Tannergren C, Turner DB, Wagner C, Weitschies W, Dressman J 2014. PBPK models for the prediction of in vivo performance of oral dosage forms. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences 57:300-321.
2. Hens B, Brouwers J, Anneveld B, Corsetti M, Symillides M, Vertzoni M, Reppas C, Turner DB, Augustijns P 2014. Gastrointestinal transfer: in vivo evaluation and implementation in in vitro and in silico predictive tools. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences 63:233-242.
3. Bergstrom CA, Holm R, Jorgensen SA, Andersson SB, Artursson P, Beato S, Borde A, Box K, Brewster M, Dressman J, Feng KI, Halbert G, Kostewicz E, McAllister M, Muenster U, Thinnes J, Taylor R, Mullertz A 2014. Early pharmaceutical profiling to predict oral drug absorption: current status and unmet needs. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences 57:173-199.
4. Augustijns P, Wuyts B, Hens B, Annaert P, Butler J, Brouwers J 2014. A review of drug solubility in human intestinal fluids: implications for the prediction of oral absorption. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences 57:322-332.
5. Khadra I, Zhou Z, Dunn C, Wilson CG, Halbert G 2015. Statistical investigation of simulated intestinal fluid composition on the equilibrium solubility of biopharmaceutics classification system class II drugs. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences 67:65-75.
6. Charman WN, Porter CJ, Mithani S, Dressman JB 1997. Physiochemical and physiological mechanisms for the effects of food on drug absorption: the role of lipids and pH. Journal of pharmaceutical sciences 86(3):269-282.
7. Holm R, Mullertz A, Mu H 2013. Bile salts and their importance for drug absorption. International journal of pharmaceutics 453(1):44-55.
8. Magee GA, French J, Gibbon B, Luscombe C 2003. Bile salt/lecithin mixed micelles optimized for the solubilization of a poorly soluble steroid molecule using statistical experimental design. Drug development and industrial pharmacy 29(4):441-450.
9. Baskaran V, Sugawara T, Nagao A 2003. Phospholipids affect the intestinal absorption of carotenoids in mice. Lipids 38(7):705-711.
10. Christensen JO, Schultz K, Mollgaard B, Kristensen HG, Mullertz A 2004. Solubilisation of poorly water-soluble drugs during in vitro lipolysis of medium- and long-chain triacylglycerols. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences 23(3):287-296.
11. Porter CJ, Trevaskis NL, Charman WN 2007. Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs. Nature reviews Drug discovery 6(3):231-248.
12. Yeap YY, Trevaskis NL, Porter CJ 2013. Lipid absorption triggers drug supersaturation at the intestinal unstirred water layer and promotes drug absorption from mixed micelles. Pharmaceutical research 30(12):3045-3058.
13. Ali MH, Moghaddam B, Kirby DJ, Mohammed AR, Perrie Y 2013. The role of lipid geometry in designing liposomes for the solubilisation of poorly water soluble drugs. International journal of pharmaceutics 453(1):225-232.
14. Reynier MO, Lafont H, Crotte C, Sauve P, Gerolami A 1985. Intestinal cholesterol uptake: comparison between mixed micelles containing lecithin or lysolecithin. Lipids 20(3):145-150.
15. Khoshakhlagh P, Johnson R, Langguth P, Nawroth T, Schmueser L, Hellmann N, Decker H, Szekely NK 2015. Fasted-State Simulated Intestinal Fluid "FaSSIF-C", a Cholesterol Containing

Intestinal Model Medium for In Vitro Drug Delivery Development. Journal of pharmaceutical sciences.
16. Armand M 2007. Lipases and lipolysis in the human digestive tract: where do we stand? Current opinion in clinical nutrition and metabolic care 10(2):156-164.
17. Clarysse S, Psachoulias D, Brouwers J, Tack J, Annaert P, Duchateau G, Reppas C, Augustijns P 2009. Postprandial changes in solubilizing capacity of human intestinal fluids for BCS class II drugs. Pharmaceutical research 26(6):1456-1466.
18. Fuchs A, Dressman JB 2014. Composition and physicochemical properties of fasted-state human duodenal and jejunal fluid: a critical evaluation of the available data. Journal of pharmaceutical sciences 103(11):3398-3411.
19. Kalantzi L, Goumas K, Kalioras V, Abrahamsson B, Dressman JB, Reppas C 2006. Characterization of the human upper gastrointestinal contents under conditions simulating bioavailability/bioequivalence studies. Pharmaceutical research 23(1):165-176.
20. Perez de la Cruz Moreno M, Oth M, Deferme S, Lammert F, Tack J, Dressman J, Augustijns P 2006. Characterization of fasted-state human intestinal fluids collected from duodenum and jejunum. The Journal of pharmacy and pharmacology 58(8):1079-1089.
21. Persson EM, Nilsson RG, Hansson GI, Lofgren LJ, Liback F, Knutson L, Abrahamsson B, Lennernas H 2006. A clinical single-pass perfusion investigation of the dynamic in vivo secretory response to a dietary meal in human proximal small intestine. Pharmaceutical research 23(4):742-751.
22. Gurantz D, Laker MF, Hofmann AF 1981. Enzymatic measurement of choline-containing phospholipids in bile. Journal of lipid research 22(2):373-376.
23. Amundson DM, Zhou M 1999. Fluorometric method for the enzymatic determination of cholesterol. Journal of biochemical and biophysical methods 38(1):43-52.
24. Armand M, Borel P, Pasquier B, Dubois C, Senft M, Andre M, Peyrot J, Salducci J, Lairon D 1996. Physicochemical characteristics of emulsions during fat digestion in human stomach and duodenum. The American journal of physiology 271(1 Pt 1):G172-183.
25. Duchateau G JH, Louter A. 2004. Plant sterol analysis in relation to functional foods. In: Dutter PD, editor Phytosterols as functional food components and nutraceuticals New York—Basel: Marcel Dekker Inc:pp 75–131.
26. Gilham D, Lehner R 2005. Techniques to measure lipase and esterase activity in vitro. Methods 36(2):139-147.
27. Weinstein DH, deRijke S, Chow CC, Foruraghi L, Zhao X, Wright EC, Whatley M, Maass-Moreno R, Chen CC, Wank SA 2013. A new method for determining gastric acid output using a wireless pH-sensing capsule. Alimentary pharmacology & therapeutics 37(12):1198-1209.
28. Persson EM, Gustafsson AS, Carlsson AS, Nilsson RG, Knutson L, Forsell P, Hanisch G, Lennernas H, Abrahamsson B 2005. The effects of food on the dissolution of poorly soluble drugs in human and in model small intestinal fluids. Pharmaceutical research 22(12):2141-2151.
29. Kleberg K, Jacobsen J, Mullertz A 2010. Characterising the behaviour of poorly water soluble drugs in the intestine: application of biorelevant media for solubility, dissolution and transport studies. The Journal of pharmacy and pharmacology 62(11):1656-1668.
30. Clarysse S, Tack J, Lammert F, Duchateau G, Reppas C, Augustijns P 2009. Postprandial evolution in composition and characteristics of human duodenal fluids in different nutritional states. Journal of pharmaceutical sciences 98(3):1177-1192.
31. Heikkila T, Karjalainen M, Ojala K, Partola K, Lammert F, Augustijns P, Urtti A, Yliperttula M, Peltonen L, Hirvonen J 2011. Equilibrium drug solubility measurements in 96-well plates reveal similar drug solubilities in phosphate buffer pH 6.8 and human intestinal fluid. International journal of pharmaceutics 405(1-2):132-136.
32. Psachoulias D, Vertzoni M, Goumas K, Kalioras V, Beato S, Butler J, Reppas C 2011. Precipitation in and supersaturation of contents of the upper small intestine after administration of two weak bases to fasted adults. Pharmaceutical research 28(12):3145-3158.

33. Vertzoni M, Markopoulos C, Symillides M, Goumas C, Imanidis G, Reppas C 2012. Luminal lipid phases after administration of a triglyceride solution of danazol in the fed state and their contribution to the flux of danazol across Caco-2 cell monolayers. Molecular pharmaceutics 9(5):1189-1198.
34. Calbet JA, MacLean DA 1997. Role of caloric content on gastric emptying in humans. The Journal of physiology 498 (Pt 2):553-559.
35. Hellstrom PM, Gryback P, Jacobsson H 2006. The physiology of gastric emptying. Best practice & research Clinical anaesthesiology 20(3):397-407.
36. Keller J, Layer P 2005. Human pancreatic exocrine response to nutrients in health and disease. Gut 54 Suppl 6:vi1-28.
37. Koo SI, Noh SK 2001. Phosphatidylcholine inhibits and lysophosphatidylcholine enhances the lymphatic absorption of alpha-tocopherol in adult rats. The Journal of nutrition 131(3):717-722.
38. Sugawara T, Kushiro M, Zhang H, Nara E, Ono H, Nagao A 2001. Lysophosphatidylcholine enhances carotenoid uptake from mixed micelles by Caco-2 human intestinal cells. The Journal of nutrition 131(11):2921-2927.
39. Makela A, Kuusi T, Seppala K, Schoder T 1998. Duodenal secretion of phospholipase A2, amylase, and bicarbonate in chronic pancreatitis. Journal of gastroenterology 33(2):260-266.
40. Geboers S, Haenen S, Mols R, Brouwers J, Tack J, Annaert P, Augustijns P 2015. Intestinal behavior of the ester prodrug tenofovir DF in humans. International journal of pharmaceutics 485(1-2):131-137.
41. Brouwers J, Tack J, Augustijns P 2007. Parallel monitoring of plasma and intraluminal drug concentrations in man after oral administration of fosamprenavir in the fasted and fed state. Pharmaceutical research 24(10):1862-1869.
42. Imai T, Ohura K 2010. The role of intestinal carboxylesterase in the oral absorption of prodrugs. Current drug metabolism 11(9):793-805.
43. Xiao D, Shi D, Yang D, Barthel B, Koch TH, Yan B 2013. Carboxylesterase-2 is a highly sensitive target of the antiobesity agent orlistat with profound implications in the activation of anticancer prodrugs. Biochemical pharmacology 85(3):439-447.